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Germline *CBL* mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia

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Author Contributions:

CMN coordinated and collected clinical data from the EWOG-MDS patients and wrote the manuscript; MWK collected clinical data, performed laboratory assays including sequencing, proliferation assays, and prepared figures; DHS performed laboratory assays including the shRNA experiments, the proliferation assays and western blots; IF collected clinical data; ME collected patients samples and performed mutational analysis on highly purified populations of blood cells; NJB contributed patient samples; SB performed ubiquitin assays; JZF contributed patient samples; TAG performed RNA isolation and cDNA sequencing; PM contributed patient samples, IS, GH, SC, PJL, CK and PGS contributed patient samples, AH provided age matched control samples from children with asthma; MS performed mutational analysis; JS, MMvH, HH, FL contributed patient samples and collected clinical data; DS collected clinical data; SA performed colony assays, performed cDNA sequencing; LC collected clinical data; RCR performed ubiquitylation assays; SSS performed ubiquitylation assays; MO supervised the ubiquitylation assays and wrote the manuscript; BSB oversaw the shRNA and cell proliferative experiments and wrote the manuscript; CF collected clinical data and performed sequencing; MLL coordinated and collected clinical and laboratory data from the U.S., oversaw all of the laboratory work, coordinated the data, and wrote the manuscript.

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Abstract

c-CBL (*CBL*) encodes a member of the Cbl family of proteins, which functions as an E3 ubiquitin ligase. We describe a dominant developmental disorder resulting from germline missense *CBL* mutations, which is characterized by constitutional anomalies that include impaired growth, developmental delay, cryptorchidism, and a predisposition to juvenile myelomonocytic leukemia (JMML). Some individuals experienced spontaneous regression of their JMML but developed vasculitis later in life. Importantly, JMML specimens from affected children show loss of the normal *CBL* allele through acquired isodisomy. Consistent with these genetic data, the common p.Y371H mutant Cbl protein induces cytokine-independent growth and constitutive phosphorylation of ERK, AKT, and S6 only in hematopoietic cells in which normal Cbl expression is reduced by RNA interference. We conclude that germline *CBL* mutations have developmental, tumorigenic, and functional consequences that are reminiscent of disorders that are caused by hyperactive Ras/Raf/MEK/ERK signaling and include neurofibromatosis type 1, and Noonan, Costello, cardiofaciocutaneous, and Legius syndromes.

Myeloproliferative neoplasms (MPNs) are clonal malignancies characterized by overproduction of immature and mature myeloid lineage cells. In particular, juvenile myelomonocytic leukemia (JMML) is an aggressive MPN of childhood characterized by malignant transformation in the stem cell compartment with clonal proliferation of progeny that variably retain the capacity to differentiate (reviewed in [1]). Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for JMML; however, relapse rates approach 30% [2]. While spontaneous remissions occur in some infants [3,4], the underlying mechanism for this is unknown.

Extensive molecular data implicate germline and somatic mutations that deregulate Ras signaling as key initiating events in JMML, with studies showing that 60% of patients harbor an oncogenic mutation in *PTPN11*, *NRAS*, or *KRAS* while another 15% have neurofibromatosis type 1 (NF1) and demonstrate loss of the normal *NF1* allele in leukemic cells [5-9]. Patients with the myeloproliferative subtype of chronic myelomonocytic leukemia (CMML), a similar MPN of adulthood, frequently acquire *NRAS*, *KRAS*, and *JAK2* mutations [10,11]. Genetically accurate mouse models recapitulate these diseases, supporting the hypothesis that hyperactive Ras is necessary and sufficient to cause MPN [12-15]. A hallmark feature of JMML and CMML is the formation of abnormally high numbers of granulocyte-macrophage colony-forming units (CFU-GM) in methylcellulose cultures containing low concentrations of GM-CSF [16,17]. Phosphorylation of the β^c chain of the GM-CSF receptor creates docking sites for adapters and signal relay molecules, resulting in activation of the Ras pathway.

Recently, we and others used high density single nucleotide polymorphism arrays to analyze blood and bone marrow specimens from patients with MPN [18-21]. These studies revealed copy-neutral loss of heterozygosity (acquired isodisomy) of a region on chromosome 11q in some cases, and subsequent studies demonstrated homozygous mutations in *CBL*. Approximately 10-15% of children with de novo JMML are estimated to harbor homozygous *CBL* mutations[20,22]. *CBL* mutations are acquired somatically in adults with MPN [18,19,21].

Children with NF1 and Noonan syndrome (NS) are predisposed to JMML [8,9,23-25], and we therefore considered the possibility that germline *CBL* mutations occur in some affected children. A review of the medical records of the 21 children with JMML found to have *CBL* mutations enrolled in the EWOG-MDS studies or treated at USCF (16 of 21 were previously included in a screen of a larger international cohort[20]) uncovered an unexpectedly high percentage with developmental delay and other congenital anomalies, which included cryptorchidism, and impaired growth (Tables 1 and 2). All children met diagnostic criteria for JMML[26,27] but six patients with a follow-up of more than seven years did not undergo transplantation for various reasons. Of these, one died of progressive JMML (D088), but the MPN improved spontaneously in five others. All of these patients continued to display variable degrees of splenomegaly in the presence of normal blood counts and exhibited the persistence of a homozygous *CBL* mutation in CD4, CD8, CD14, and CD19 sorted cells from their peripheral blood at last follow-up. In addition, four of these patients have developed clinical signs consistent with vascular pathology, including optic atrophy, hypertension, and an acquired cardiomyopathy; one was diagnosed with Takayasu arteritis, type III by angiography (Figure 1a). Another patient (D256) developed an intracranial germinoma harboring the same homozygous *CBL* mutation as in his bone marrow. Of note, among the patients treated with HSCT, there was a high rate of conversion to stable mixed chimerism (8/11 patients with available data) (Table 1).

We analyzed normal tissues from 17 of these children and detected a heterozygous *CBL* mutation in each. Mutational analysis of parental DNA was informative in 13 families and confirmed autosomal inheritance of a *CBL* mutation in seven (Figure 1b, 1c, Table 2, and Figure S1). Patients UPN1333 and UPN1125 were from large pedigrees in which several individuals had died of JMML (Figure 1b and c).

The proband in family 1 (UPN1333, V:1) (Figure 1a) was referred after transplantation for JMML. Initially diagnosed at 7 months of age, he received his first HSCT at age 13 months and then developed mixed chimerism 6 months later. A blood sample displayed a homozygous *CBL* mutation at c.1111T>C (Y371H). Importantly, analysis of buccal swab DNA revealed a heterozygous lesion. Both maternal relatives died from progressive JMML. Peripheral blood or buccal swabs from extended family members revealed multiple heterozygous individuals (Figure 1b). Interestingly, detailed medical history from the affected maternal great-grandmother revealed a history of infant leukemia characterized by a high white blood cell count and splenomegaly that resolved spontaneously. Sorted B (CD19) and T (CD3) cells, and granulocytes from her peripheral blood currently display a heterozygous c.1111T> C. Leukemia cells from UPN1333 displayed a classic pattern of GM-CSF hypersensitivity (Figure 1d) and increased phosphorylation of STAT5 in response

to low doses of GM-CSF [28]. Peripheral blood mononuclear cells from his heterozygous mother did not display either of these features, suggesting that homozygosity for the mutant *CBL* allele is essential for these cellular behaviors (data not shown).

The proband in family 2 (UPN1125, IV:3) was a girl diagnosed at 15 months of age with JMML. She also harbored a homozygous c.1111T>C *CBL* mutation in her bone marrow. Family history revealed that her mother had two male first cousins who were diagnosed with JMML and died before age 10. The first boy had archived frozen liver tissue available from autopsy and demonstrated a heterozygous c.1111T>C mutation. Interestingly, he also developed clinical signs and laboratory values consistent with small vessel vasculitis prior to his death. Her mother of UPN1125 was found to carry a heterozygous *CBL* mutation (Figure 1c).

Three patients in our series displayed homozygous splice site mutations (Table 2, I066, D647, and D347). RT-PCR demonstrated several new splice products arising from these mutations (Figure 2); most notable are the 2 splice site variants that either delete the entirety of exon 8 (D347) or exon 9 (I066 and D647) (Figure S2) or retain an interstitial intron (e.g. intron 7 for D347). Each of the deletion splice variants encodes a protein that is predicted to lack critical regions of the linker and RING finger domains, while the intron 7 retention introduces a premature stop codon to abort translation upstream of the RING finger domain.

To investigate the functional properties of the variant mutant Cbl proteins encoded by homozygous point mutations, we first studied the effect of the common p.Y371H substitution on the growth of primary hematopoietic cells from murine fetal liver. This system reproduces the hypersensitivity to GM-CSF that is characteristic of JMML [16,29]. Fetal liver cells transduced with retroviral vectors expressing wildtype or mutant Cbl proteins demonstrated no increased sensitivity to GM-CSF (Figure 3a). Similarly, expression of p.Y371H Cbl in a BaF3-EpoR cell line did not confer cytokine independence (Figure 3b). Based on the observation that JMML cells invariably lose the normal *CBL* allele and on recently published data[21], we reasoned that reduction of normal Cbl expression might be mandatory to deregulate hematopoietic growth. Therefore, we introduced a short hairpin RNA, which markedly reduced the expression of murine Cbl in BaF3-EpoR cell lines (Figure 3c). We next transduced these cells with a series of wildtype and mutant human constructs and demonstrated strong expression of exogenous Cbl (Figure 3c). In this context, we observed cytokine independent proliferation (Figure 3d) upon expression of p.Y371H Cbl and a known murine oncogenic Cbl protein (70Z)[30-32]. The 70Z oncogenic protein deletes out 17 amino acids located from position 366-382 in the linker domain. Furthermore, the p.Y371H and 70Z transduced cells demonstrated hypersensitivity to increasing concentrations of human EPO (Figure 3e), mimicking the growth factor hypersensitivity seen in JMML (Figure 1d). In cells depleted of endogenous Cbl and expressing exogenous p.Y371H and 70Z Cbl proteins, we observed constitutive phosphorylation of ERK, AKT and S6 in cells deprived of cytokine. We also found heightened responses to low dose EPO (Figure 2f).

In order to determine if mutant Cbl proteins retain E3 ligase activity, we assessed their ability to promote ubiquitylation of a known Cbl substrate. Soon after activation, the

epidermal growth factor receptor (EGFR) undergoes Cbl-dependent polyubiquitylation and proteosomal degradation. HA-Cbl(p.Y371H)-expressing HEK293 cells exhibited markedly elevated levels of phosphorylated EGFR upon EGF stimulation in comparison to HA-Cbl(WT)-expressing counterpart (Figure 4a), which indicates a possible defect in clearance of pEGFR by Cbl(p.Y371H). Cbl(p.C384R) mutant exhibited a similar defect in pEGFR clearance (data not shown). Upon polyubiquitylation of targets, Cbl promotes its own polyubiquitylation and subsequent auto-degradation. Consistent with this model, levels of Cbl(WT), but not Cbl(p.Y371H or p.C384R), rapidly diminished following EGF treatment in the absence of proteasome inhibitor MG132 (Figure 4a, b and data not shown,). However, Cbl(WT) levels were markedly stabilized in the presence of MG132 while Cbl(p.Y371H) levels remained relatively high irrespective of MG132 (Figure 4b), which suggest that Cbl mutants p.Y371H and p.C384R have an inherent defect in E3 function. Consistent with this notion, Cbl(WT) promoted robust polyubiquitylation of pEGFR while p.Y371H and p.C384R showed diminished capacity to polyubiquitylate pEGFR in an in vitro ubiquitylation assay (data not shown), similar to that of Cbl(70Z), which is known to be defective in E3 ligase activity, and in concordance with recently published data by Sanada et al [21], who demonstrated that p.Y371S and p.Q367P mutants detected in human patients also have diminished ubiquitylation activity. The Y371 residue most commonly affected in germline *CBL* mutations has been the focus of extensive biochemical analysis [21]. This literature supports a key role for Y371 in maintaining the integrity of the alpha-helical structure of the linker region, which plays a critical role in substrate specificity. Interestingly, Y371 has been found to be phosphorylated despite its predicted location away from the protein surface. Substitution of this residue with phenylalanine results in a nononcogenic form of the protein, which lacks E3 ligase activity. Conversely substitution with a glutamate constitutively activates E3 ligase activity [33,34].

In most ways, *CBL* appears to function as a classic tumor suppressor gene in this cohort, with germline heterozygosity predisposing to neoplasia upon reduction to homozygosity in target tissues. However, the predominance of specific missense mutations makes *CBL* distinct from most tumor suppressor genes such as *RB* and *NF1*, which typically demonstrate more severe loss of function mutations such as deletion or protein truncation. This suggests that the mutant Cbl proteins retain an essential biochemical function [20,21]. It is further supported by our data showing that exclusive expression of a mutant *CBL* allele has positive effects on cytokine signaling and proliferation. The specific disruption of E3 ligase activity may leave intact adapter functions, resulting in a relative imbalance of CBL's positive and negative roles in signal transduction. Sanada et al hypothesized that this may result in inhibitory effects on Cbl-b, a related family member[21]. This is similar to what has been reported for p53, a classic tumor suppressor gene with specific gain of function mutations in the context of loss of heterozygosity [35,36]. Beyond missense mutations, truncated Cbl proteins are also known to confer transforming effects by countering the negative action of full-length Cbl on RTK signaling. The *Drosophila* analogue of the mammalian v-Cbl oncogene (i.e. Dv-Cbl), for example, functions as oncogenic dominant-negative variant whose expression results in vivo in enhanced signaling of the EGFR cascade and cooperates with activating mutations in the Ras pathway to ultimately produce melanotic tumors [37].

In addition, clinical data reveals that some patients may experience spontaneous resolution of JMML but go on to develop clinical features consistent with vasculitis and other autoimmune phenomena later in life. Interestingly, autoimmunity has also characterized genetically engineered mouse strains with Cbl mutations. *Lck-Cre*$^{+}$ c-*Cbl*$^{flox/flox}$ *Cbl-b*$^{-/-}$ mice, which delete c-Cbl in T cells, develop severe vascular lesions with massive infiltration of T-cells and high concentrations of anti-double stranded DNA antibodies [38]. Furthermore, these T cells are hypersensitive to T cell receptor signaling and display prolonged ERK phosphorylation. Similarly, *Cd19-Cre*$^{+}$ c-*Cbl*$^{flox/flox}$ *Cbl-b*$^{-/-}$ mice that delete c-*Cbl* in B cells develop a lupus-like syndrome associated with perivascular infiltration and hyperactivation of B cell receptor signaling [39]. In mice, loss of *Cbl-b* is required for these phenotypes. Oncogenic c-*Cbl* proteins thus may inhibit *Cbl-b in vivo*, resulting in a functional loss of both *Cbl* and *Cbl-b*, which in turn contributes to dysregulated lymphocyte signaling and subsequent vasculitis. Indeed, the redundant role of c-*Cbl* and *Cbl-b* was further explored by Sanada, et al, who demonstrated the inhibitory effects of c-*Cbl* p.Y371S on wildtype *Cbl-b* [21]. It is of clinical interest that patients with JMML and homozygous *CBL* mutations who undergo HSCT are not known to develop vasculitis later in life, implying that a normal immune system is critical to preventing this late manifestation.

We describe a new syndrome in which affected children display several congenital anomalies that overlap with NF1, NS, and Legius, suggesting that the affected proteins converge on the Ras/MAPK pathway. Indeed, several Ras/MAPK pathway proteins regulate developmental programs in multiple species - for instance, the Drosophila homologs of each of these genes--*CBL* (D-cbl), *PTPN11* (csw), *NF1*, and *SPRED* perform critical functions for growth and patterning [40-43].

Patients with germline *CBL* mutations are at increased risk of developing JMML, which may follow an aggressive clinical course or resolve without treatment. Some affected individuals develop vasculitis later in life. The *CBL* mutations found in JMML can arise de novo or can be transmitted through the germline, and human leukemia samples invariably show loss of the normal *CBL* allele. Consistent with this tumor suppressor function, JMML-associated Cbl proteins confer cytokine hypersensitivity in transduced BaF3-EpoR cells in the absence of wildtype Cbl, have defective E3 ligase activity, and constitutively activate key Ras effector pathways. The role of aberrant Cbl signaling in vasculitis remains to be determined, and it will be particularly interesting to investigate if patients with germline *CBL* mutations who have been cured of JMML after HSCT remain at risk of developing vasculitis. It is also of great interest that some of these patients continue to display homozygous *CBL* mutations in their peripheral blood despite having improved their blood counts. Finally, our data provide strong evidence that Cbl is a key negative regulator of Ras signaling networks in hematopoietic cells and it will be important to identify key targets of the Cbl ubiquitin ligase and to uncover other biochemical mechanisms involved in growth control.

METHODS

Subjects

Patients were diagnosed and treated either in Europe under the auspices of the European Working Group of Myelodysplastic Syndromes in Childhood (EWOG-MDS) or enrolled as research subjects at the University of California, San Francisco. The Committees on Human Research at each of the institutions in EWOG-MDS, as well as UCSF approved these studies. Informed consent was obtained from parents or guardians, and in the case of pedigree analysis, all screened relatives. Family and clinical histories were reviewed, as were physical exams at diagnosis.

Mutation Screening

Bone marrow or peripheral blood samples at diagnosis were obtained. Mononuclear cells were isolated using standard Histopaque 1111. Buccal swabs, fibroblasts, or tissues unaffected by tumor were also obtained when available. Genomic DNA was extracted using PureGene reagents (Qiagen, Foster City, CA). Patients were screened for mutations in *CBL*, *NRAS*, *KRAS*, and *PTPN11* as previously described[6,7,20].

RNA was prepared according to usual methods. cDNA was generated using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). Splice variants were identified through PCR using an annealing temperature of 58 °C and the following primers: 5'-TTGAGGGAACACATACTCGCT-3' and 5'-TATGTTACTGCTGATGGGAACA-3'. Splice variants were gel extracted using the QIAquick Gel Extraction Kit (Qiagen). The resulting fragments were subcloned using the TOPO® TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Positive colonies were picked, mini prepped using the QIAprep Spin Miniprep Kit (Qiagen), and sequenced with standard M13 Forward (-20) and M13 Reverse primers.

Cbl and shRNA expression constructs

For the CFU-GM experiments, Gateway technology (Invitrogen) was used to clone WT and mutant *Cbl* cDNAs into the murine stem cell virus (MSCV) backbone containing a green fluorescent protein (GFP) cassette driven by an internal ribosome entry site (IRES) downstream of the *Cbl* sequence (pMIG). The human WT and 70Z Cbl plasmids were a kind gift from Hamid Band [44]. For the subsequent experiments using Ba/F3 cells, the same *Cbl* cDNAs were cloned into an MSCV-IRES-Venus (pMIV) backbone to allow for co-transduction with the GFP-tagged shRNA. MiR30 based shRNA sequences targeting murine *Cbl* were graciously designed by Johannes Zuber and Scott Lowe. We selected one of these putative sequences (cbl.2364) and custom ordered a single 110-bp oligonucleotide (Bioneer) to serve as a template for PCR amplification. PCR products were digested with *XhoI* and *EcoRI* and ligated into the LTR-driven MiR30 SV40-GFP (LMS) MSCV-based vector (also graciously provided by the Lowe lab) to produce the LMS-2364 retrovirus encoding the *Cbl* shRNA. The sequence for cbl.2364 is TCGAGAAGGTATATTGCTGTTGACAGTGAGCGATACCTATGAAGCGATGTATAATAGTGAAGCCACAGATGTATTATACATCGCTTCATAGGTACTGCCTACTGCCTCGG.

Hematopoietic progenitor assays

All experimental procedures involving mice were reviewed and approved by the UCSF Committee on Animal Research. These assays were performed as described previously using murine fetal liver cells transduced with MSCV-*Cbl*-IRES-GFP retroviruses engineered to express WT or mutant Cbl proteins. For the human CFU-GM assays, mononuclear cells from peripheral blood or bone marrow were plated in MethoCult H4230 (StemCell Technologies), supplemented with recombinant human GM-CSF (Peprotech) and counted 14 days later as described previously [28]. For the CFU-GM assays, GFP-positive cells were sorted using a FACS Aria (BD Biosciences) and then seeded in methylcellulose medium (M3231; StemCell Technologies) [45], supplemented with recombinant murine GM-CSF (Peprotech). Colonies were counted by indirect microscopy after 8 days.

Cell Viability and Proliferation assays and Western blots

Murine pro-B Ba/F3 cells were transduced with MSCV-EpoR-IRES-puro as described previously [46]. These cells were maintained in RPMI-1640 with 10% FCS (HyClone), penicillin, streptomycin, L-glutamine, 10 ng/ml puromycin (Calbiochem), and 10 μg/ml murine IL-3 (Peprotech).

The Ba/F3-EpoR cells were transduced with the LMS-2364 construct or the LMS vector alone. GFP-positive cells were sorted on a FACS Aria (BD Biosciences) and then transduced with the MSCV-*Cbl*-IRES-Venus retroviruses expressing WT or mutant *Cbl* proteins. Cells positive for both GFP and YFP expression were sorted on the FACS Aria and studied in proliferation and Western blot assays.

For the proliferation assays, cells were washed 3x and then cultured for 6 hours in cytokine-free media before being plated in 6-well plates at a density of 500,000 cells/ml at increasing doses of hEPO (R&D Systems). Growth was monitored every other day using a ViCell cell counter (Beckman Coulter).

For Western blot analysis, cells were washed 3x and cultured for 6 hours in cytokine-free media before being stimulated for 15 minutes with increasing doses of hEPO (R&D Systems). Whole-cell lysates were blotted and probed with the following antibodies: anti-phospho-p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204, cat. 9101), anti-phospho-AKT (S473, cat. 4060), anti-phospho-S6 (Ser235/236, cat. 2211) (all from Cell Signaling Technology); anti-phospho-STAT5 (cat. 44-390G) (Invitrogen) and anti-alpha-tubulin (Abnova). ERK1/2 (cat. 9102), AKT (cat. 9272), S6 (2217), and STAT5 (9363) antibodies were from Cell Signaling Technology.

HEKCells

HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada) at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies

Mouse monoclonal antibodies against HA (12CA5) and α-tubulin were obtained from Boehringer Ingelheim (Laval, QC, Canada) and Sigma (Milwaukee, WI), respectively. Rabbit polyclonal antibodies against pEGFR, EGFR and ubiquitin were obtained from Upstate (Temecula, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and DAKO Canada (Mississauga, ON, Canada), respectively. EGF ligand was obtained from Sigma (Milwaukee, WI). MG132 proteasome inhibitor was obtained from Boston Biochem (Cambridge, MA).

Plasmids

Plasmids encoding HA-CBL(WT, 70Z, Y371H, C384R) were subcloned into the pcDNA-DEST4.0 vector via Gateway Cloning technology (Invitrogen, Carlsbad, CA) and confirmed by DNA sequencing. Plasmid encoding EGFR was generated as previously described [47].

Immunoblotting and immunoprecipitation

Immunoprecipitation and immunoblotting were performed as described previously [48]. Cells were lysed in EBC buffer (50mM Tris pH 8.0, 120mM NaCl and 0.5% NP-40) supplemented with protease and phosphatase inhibitors (Roche, Laval, QC, Canada). Cell lysates were immunoprecipitated with indicated antibodies in the presence of Protein-A agarose beads (Repligen, Waltham, MA). Bound proteins were washed five times with NETN buffer (20mM Tris pH 8.0, 120mM NaCl, 1mM EDTA, and 0.5% NP-40), eluted by boiling in sodium dodecyl sulfate (SDS)-containing sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

In vitro ubiquitylation assay

In vitro ubiquitylation assay was performed as described previously [49].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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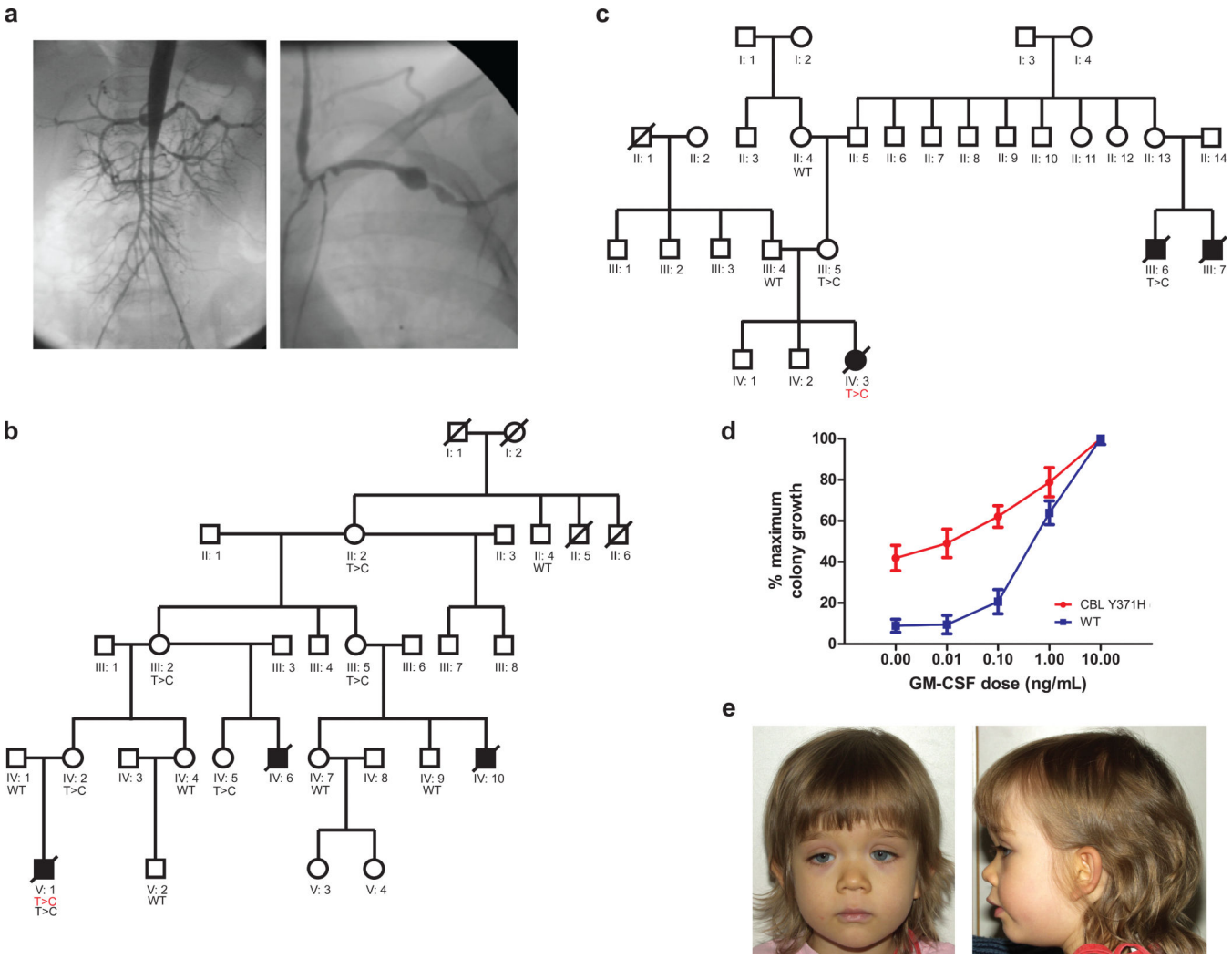

Figure 1. Germline mutations in *CBL* can be inherited in an autosomal dominant fashion and are associated with a phenotype, GM-CSF hypersensitivity and vasculitis

Panel (a) demonstrates the angiograms from the aorta and left subclavian artery from patient D048 nine months after the diagnosis of Takayasu arteritis type III. Panel (b) The family tree of UPN1333 is shown in panel a, where the diseased bone marrow of UPN1333 displayed a homozygous *CBL* c.1111T>C (red) mutation as well as a heterozygous lesion from his buccal swab (black). Only women appear to be heterozygote carriers, and only boys appear to be affected by JMML in this family. Panel (c) The bone marrow of UPN1125 demonstrated a homozygous *CBL* mutation—her mother (III:5) is a known carrier, and she had two male cousins dying from JMML (III:6, III:7). Panel (d) demonstrates a classic GM-CSF hypersensitivity response on a colony assay for patients with CBL mutations (n=3) versus normal (n=13). Error bars represent standard error of the mean (s.e.m.) Panel (e) shows one toddler (D703) diagnosed with JMML and a homozygous mutation at p.C384R. She displays frontal bossing, downslanting palpebral fissues, hypertelorism, and a low nasal bridge. Photographs of her father, who harbors a heterozygous mutation at p.C384R, are included in Figures S1, panel d. Of note, both father and daughter also display bilateral ptosis.

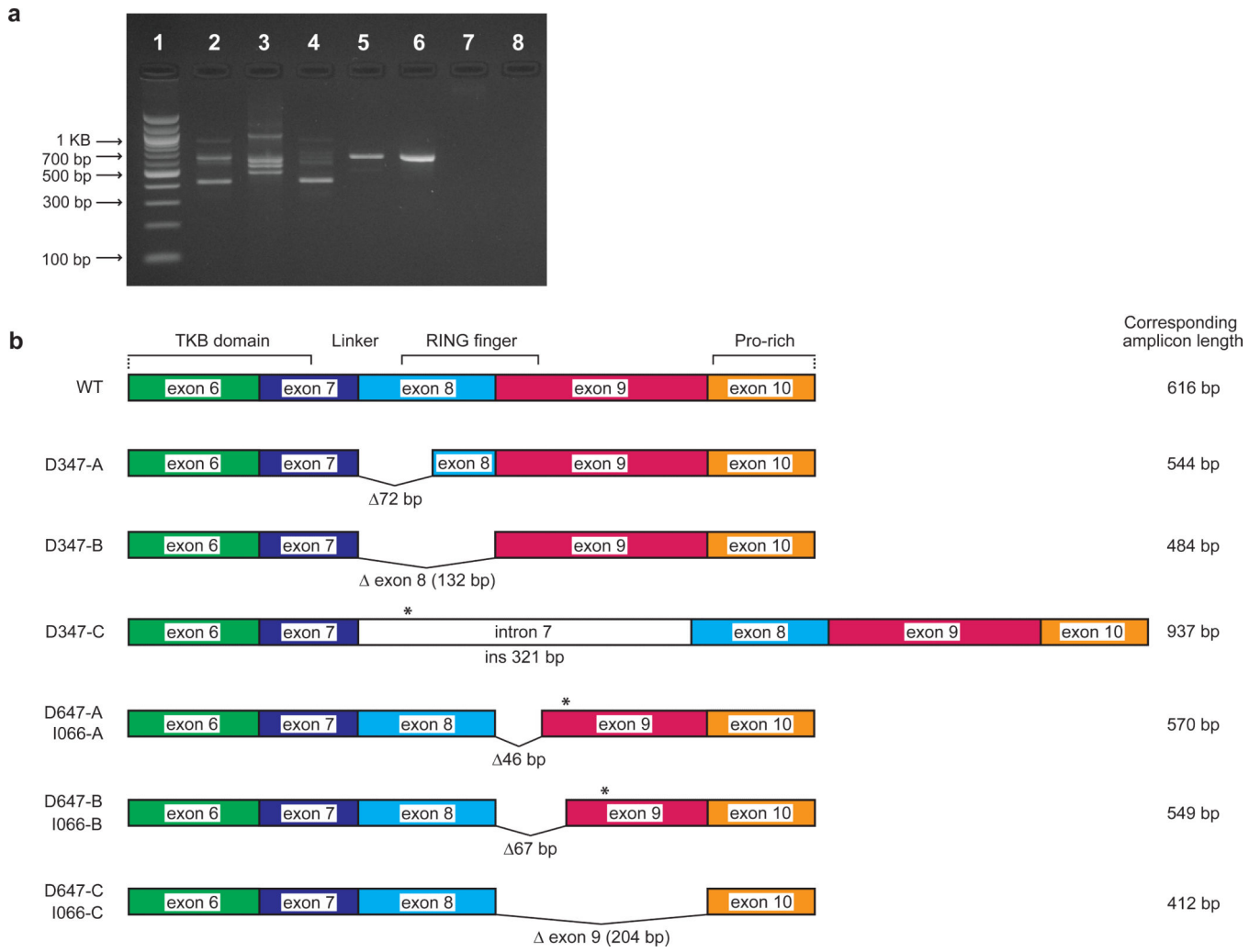

Figure 2. Consequences of splice site mutations in cDNA from individuals D347, 647, and I066

Panel (a) RT-PCR using an exon 6 forward primer and an exon 10 reverse primer on cDNA generated from these patients. The wildtype amplicon is 616 base pairs long. Lane 1: MW ladder, Lane 2: I066, Lane 3: D347, Lane 4: D647, Lane 5: *CBL* point mutant, Lane 6: HM2833 *CBL* wildtype, Lane 7: Genomic DNA control, Lane 8: no template control. Panel (b) is a schematic representation of the splice site variants detected either recurrently (D347) or that were shared by I066 and D647. Sequences are shown in Figure S2. Deletions of base pairs are indicated by Δ# of base pairs and insertions by ins # base pairs. Premature stop codons are indicated by asterisks (*).

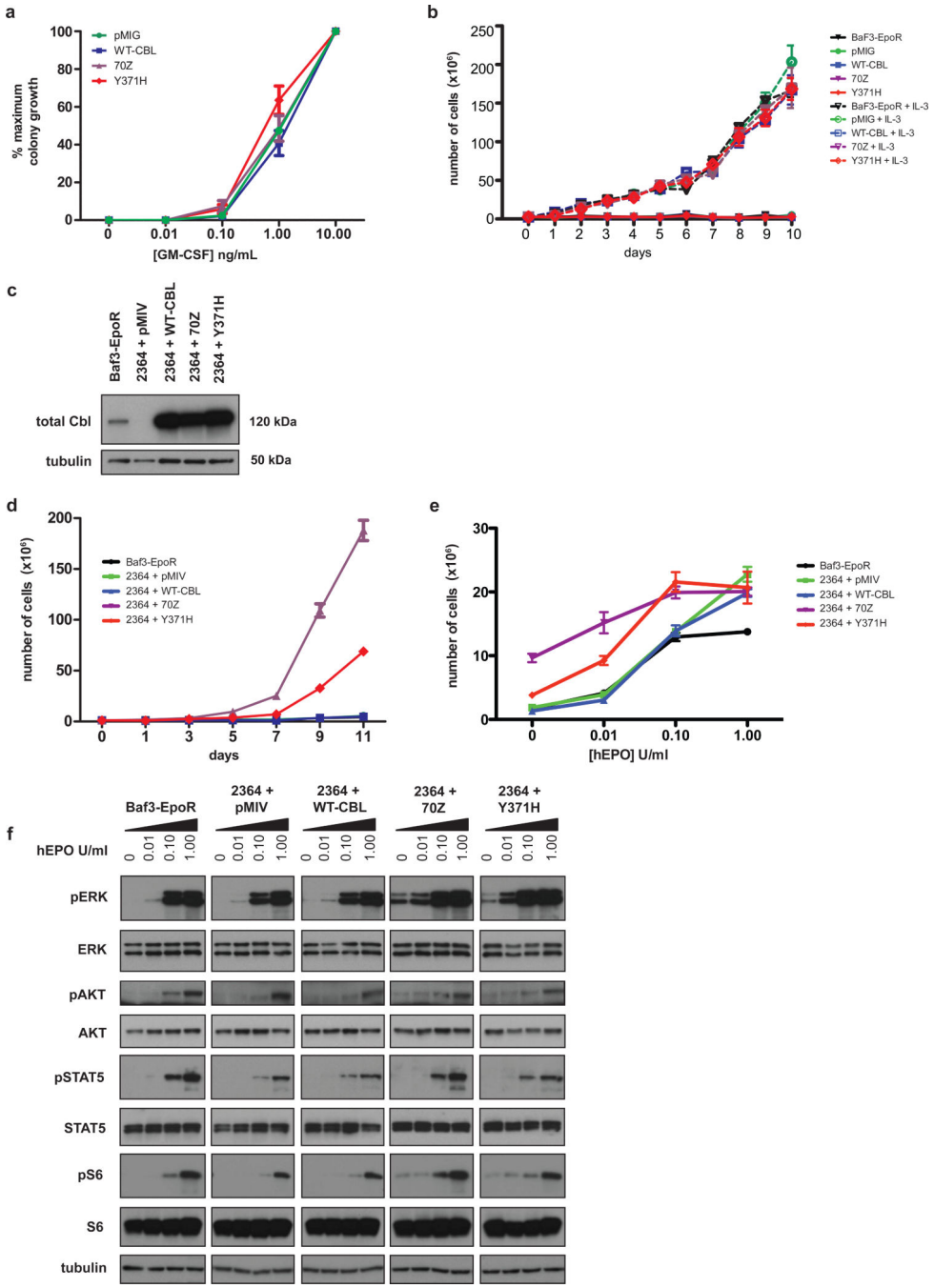

Figure 3. p.Y371H does not confer cytokine sensitivity or cytokine independent growth until silencing of murine Cbl

Panel (a) Transduction of p.Y371H or the known murine oncogeneic mutant 70Z in wildtype hematopoietic cells from fetal liver, did not confer hypersensitivity to GM-CSF, nor did expression of these mutants in BaF3-EpoR cells result in cytokine independent growth (b). Panel (c) An shRNA to murine Cbl (cbl.2364) demonstrated near complete shutdown of expression in BaF3-EpoR cells by Western blot with re-expression upon introduction of the human WT, 70Z, or p.Y371H in these same cell lines. Panel (d) Both the p.Y371H and 70Z Cbl conferred cytokine independent growth in the presence of cbl.2364.

Controls included the Venus vector pMIV and BaF3-EpoR. Error bars for triplicate replicates (s.e.m.) are shown and when not visible, indicate tight clustering. Using a paired t-test: day 7 comparing 2364+ WT-Cbl versus 2364+p.Y371H, p-value= 0.017, and at day 9: p-value <0.001. Panel (e) Serial transduction of the hairpin (2364) and p.Y371H or 70Z constructs also conferred hypersensitive growth after assessing cell proliferation on day 5 in increasing concentrations of Epo. Using a paired t-test at each concentration of Epo when comparing 2364+WT-Cbl versus 2364+p.Y371H: Epo 0 unit/ml: p = 0.036, Epo 0.01 units/ml: p= 0.0015, Epo 0.1 units/ml: p= 0.029, Epo 1 unit/ml (saturating dose) P= 0.697. Panel (f) Both the p.Y371H and 70Z containing cells demonstrated activation of pERK, pAKT, and pS6 in the absence of Epo or in low dose 0.01 unit/mL of Epo in comparison to negative controls. All cell proliferation work was done in triplicate.

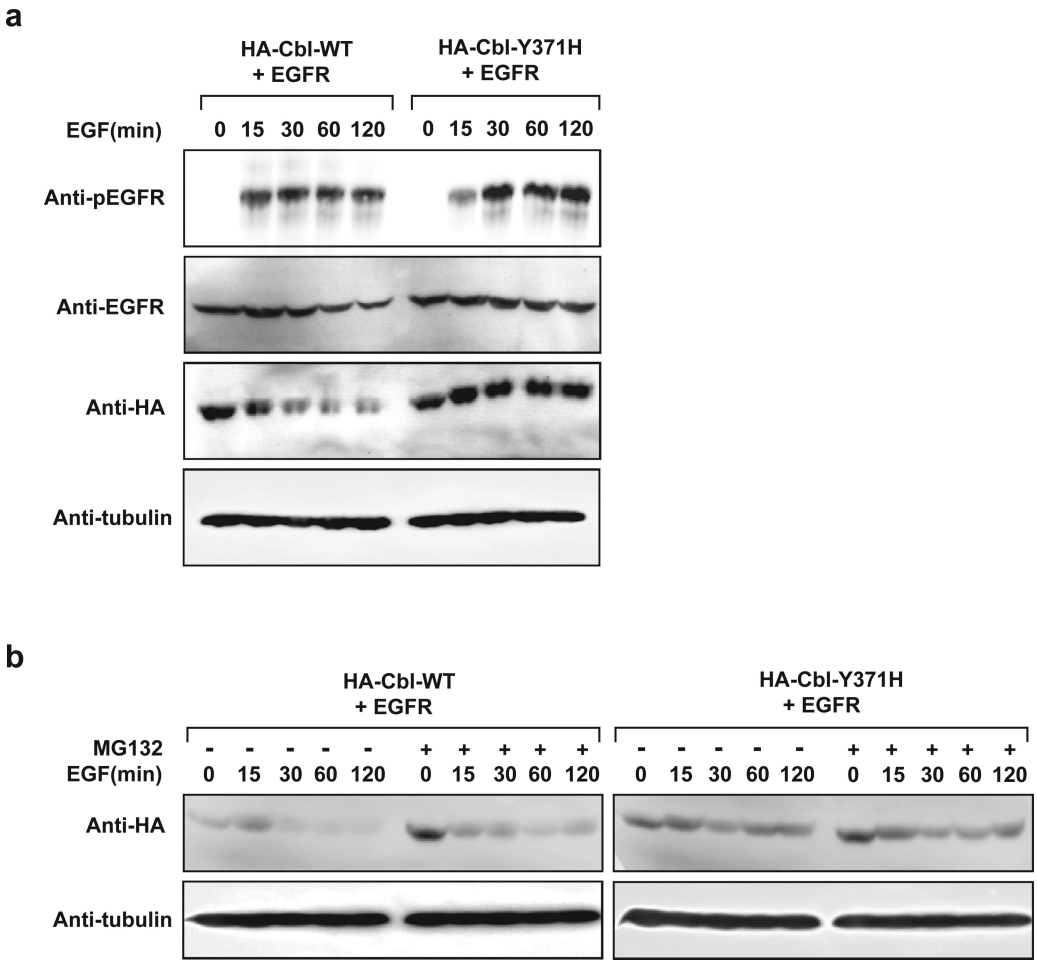

Figure 4. Cbl mutant proteins exhibit prolonged protein turnover and are associated with increased phosphorylated EGFR levels upon EGF stimulation

Panel (a) HEK293 cells transfected with plasmids encoding EGFR in combination with HA-Cbl(WT) or HA Cbl(p.Y371H) were serum starved for 18 h followed by 15 min EGF (50 ng/ml) stimulation. Cells were then washed and maintained in serum-free media for the indicated periods of time. Equal amounts of whole cell extracts were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. Panel (b) HEK293 cells transfected with plasmids encoding EGFR in combination with HA-Cbl(WT) or HA-Cbl(p.Y371H) were serum starved for 18 h followed by 15 min EGF (50 ng/ml) stimulation. Cells were then washed and maintained in serum-free media with (+) or without (-) MG132 for the indicated periods of time. Equal amounts of whole cell extracts were resolved on SDS-PAGE and immunoblotted with the indicated antibodies.

Table 1

Hematological Features at Diagnosis, Hematopoietic Stem Cell Transplantation and Current Status in 21 Children with homozygous *CBL* Mutations in JMML Cells

ID	Sex	Age and Hematological Features at Diagnosis						HSCT		Last fo low-up	
		Age (years)	WBC (×10^9/L)	% blasts PB/BM	Platelets (×10^9/L)	Spleen Size[a]	Karyotype	Age (years)	MC / CC	Alive/Dead	Age (years)
A053	M	0.1	112	1 / 2	20	9	normal	-	-	A	18.2
A054	F	1.1	36	1 / 4	95	7	normal	-	-	A	17.7
D256	M	3.6	13	1 / 6	18	10	normal	-	-	A	13.0
D048	F	1.6	23	0 / 2	46	9	normal	-	-	D	9.9
D389	M	0.6	14	0 / 6	67	8	normal	-	-	A	7.5
D088	M	0.8	46	3 / nd	32	12	normal	-	-	D	7.3
NL075	F	1.5	55	5 / 2	55	12	normal	1.8	MC	A	7.3
CZ039	M	1.1	29	3 / 4	284	6	normal	1.2	MC/CC[c]	A	7.0
D451	M	0.7	27	2 / 3	86	6	normal	1.2	MC/CC[d]	A	6.2
D647	F	2.5	67	1 / 0	117	6	normal	3.9	CC	A	6.1
I066	F	5.0	21	2 / 10	33	5	normal	5.7	MC	D	6.0
SC084	M	1.4	33	0 / 0	33	13	normal	2.0	NA	A	4.8
D104	M	2.2	27	0 / 2	232	5	normal	-	-	D	3.9
D347	M	0.9	72	4 / 6	48	10	45,XY,−16	1.2	MC	D	3.4
D703	F	1.4	27	2 / 1	15	4	46,XX+der (8)	1.7	MC	A	3.0
UPN1333	M	0.6	36	3 / 4	46	4	normal	1.1	MC	D	2.6
D251	F	0.6	31	0 / 20	37	4	normal	1.1	CC	D	2.4
UPN1778	M	1.3	22	0 / 1.5	61	5	normal	-	-	A	2.0
UPN1125	F	1.3	196	4 / 5	47	4	normal	1.7	CC	D	2.1
D774	F	1.5	22	0 / 4	266	3	normal	-	-	A	1.5
UPN1241	F	0.6	43	5 / 3	62	4	normal	1.1	MC	D	1.5

ID, patient identification. M, male. F, female. WBC, white blood count. PB, peripheral blood. BM, bone marrow. HSCT, -hematopoietic stem cell transplantation. A, alive. D, dead. MC, mixed chimerism. CC, complete chimerism. NA, not analyzed.

[b] All patients had received a myeloablative preparative regimen prior to HSCT. One patient with MC died of progressive disease (UPN 1333), the other deaths were due to transplant related toxicities.

[a] in cm below costal margin in left midclavicular line

[c] patient converted back to CC after donor lymphocyte infusion

[d] patient converted back to CC without therapy

Table 2

CBL Mutation and Non-Hematological features in 21 Children with JMML

ID	*CBL* Mutation				Café au lait spots[m]	JXG	Cryptorchism	Growth < 3rd percentile	Dev. delay	Hearing loss	Optic atrophy	Hypertension[n]	Cardiomyopathy
	Mutation	Germ	Mo	Fa									
A053[a]	p.C396R	+	-	NA	-	-	-	-	+	+	+	+	+
A054	p.Y371H	+	+	NA	-	+	female	-	-	-	-	-	-
D256[b]	p.W408R	+	-	-	+	+	-	+	+	-	+	+	-
D048[c]	p.C384R	NA	NA	NA	-	-	female	+	+	-	+	+	+
D389[d]	p.Y371C	+	-	+	+	-	+	-	+	+	+	+	+
D088	p.Y371D	NA	NA	NA	-	-	+	+	-	-	-	-	-
NL075	p.Y371H	+	-	-	-	+	female	-	-	-	-	-	-
CZ039	p.C404R	+	-	-	-	-	-	-	-	-	-	-	-
D451	p.Y371H	+	-	-	-	-	+	-	+	-	-	-	-
D647	c.1228-2A>G splice site	+	-	-	-	-	female	+	+	-	-	-	-
I066	c.1228-2A>G splice site	NA	NA	NA	-	-	female	-	-	-	-	-	-
SC084	p.Y371H	+	+	-	-	-	-	-	+	-	-	-	-
D104[e]	p.C384R	NA	NA	NA	-	+	-	-	-	-	-	-	-
D347[f]	c.1096-1G>C splice site	+	-	NA	+	+	+	+	+	-	-	-	-
D703	p.C384R	+	-	+	-	-	female	-	-	-	-	-	-
UPN1333	p.Y371H	+	+	-	-	-	-	-	+	+	-	-	-
D251[g]	p.L380P	+	NA	NA	-	+	female	+	+	-	-	-	-
UPN1778[h]	p.Y371N	+	-	NA	-	-	-	-	-	-	-	-	-
UPN1125	p.Y371H	+	+	-	-	-	female	-	-	-	-	+	-
D774[k]	p.H398R	+	+	-	-	-	female	-	-	-	-	-	-
UPN1241	p.Y371H	+	-	-	+	-	female	-	-	-	-	-	--

ID, patient identification, Germ, germline. Mo, mother. Fa, Father. NA, not analyzed. JXG, juvenile xanthogranuloma. Dev., developmental.

[a] autoimmune thyreoditis with anti-thyreoglobulin antibodies; echocardiography with mitral insufficiency grade II, myocardial hypertrophy, hypoelastic and hypoplastic ascending aorta and arch of aorta

[b] Diagnosis of intracranial germinoma at age 9 years with homozygous CBL mutation in the brain tumor

[c] Diagnosed with Takayasu arteritis, type III, died 9 months after diagnosis of vasculitis and stenting of major arteries

[d] Father with history of Hodgkin lymphoma

[e] Sudden death at home

[f] Died of cerebral hypoxia, also had a history of supraventricular tachycardia

[g] Prior to HSCT, was diagnosed with erythroderma, post-HSCT: toxic epidermal necolysis, acute liver failure, liver Tx, subtotal brain infarction, apallic syndrome

[h] Pectus excavatum

[k] Older sister with heterozygous mutation

[m] Patients indicated by + have 1-2 café au lait spots, there was no patient with 6 or more café au lait spots

[n] Measurements of blood pressure following HSCT were excluded from analysis